Host proteins are associated with adenovirus specific mRNA in the cytoplasm

Walter J. van Venrooij, Thieu Riemen and Chris A.G. van Eekelen

Department of Biochemistry, University of Nijmegen, Geert Grooteplein Noord 21, 6525 EZ Nijmegen, The Netherlands

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1. INTRODUCTION

In eukaryotic cells mRNA and its precursors are always associated with proteins to form ribonucleoprotein complexes. The major protein components present in isolated cytoplasmic mRNA–protein complexes have M_r of approximately 73 000 and 52 000. The larger protein has been shown to be associated with the poly(A)-region of cytoplasmic mRNA [1,2]. Although there probably are more mRNA-associated proteins, the published reports on their variations and diversity remain a matter of considerable dispute. One reason for the reported discrepancies is the fact that the methods used to isolate mRNP complexes have the acknowledged disadvantage that non-specific binding of proteins to the RNA during cell fractionation may occur. On the other hand, conditions which minimize non-specific adsorption mostly promote dissociation of messenger-associated proteins from the RNA. Another problem is created by the fact that some proteins present in mRNP particles might not be directly associated with the RNA but, for example, via protein–protein interactions. Particular difficulties arise when viral mRNA–protein complexes have to be purified from mixtures in which host mRNP complexes are present as well. Reliable separations between viral and host mRNA–protein complexes have never been carried out. Thus, most reports on the composition of viral mRNA–protein particles focused on differences in the composition of viral and host complexes [3–5].

We have used cross-linking in vivo by means of ultraviolet irradiation of intact cells as the method of choice to avoid all these problems. The advantages of this method, in detail described by Wagenmakers et al. [6] and by Greenberg [7] can be summarized as follows: (1) Only proteins closely associated with RNA can be cross-linked with the RNA (for references see [6]). (2) Since the irradiation is performed on intact cells only proteins interacting in vivo with RNA are covalently linked. (3) Isolation of the covalent RNA–protein complexes can be performed under conditions that exclude co-purification of non-specifically associated proteins. (4) Sequence-specific mRNP complexes, for example virus-specific complexes, can be isolated via hybridization with specific DNA probes coupled to cellulose or Sepharose beads. Such an approach has been used recently to isolate adenovirus specific hnRNA–protein complexes [8]. (5) When the RNA is prelabeled in vivo, the cross-linked proteins that directly interact with the RNA can be detected after gel electrophoresis by fluorography owing to the radioactive nucleotides covalently linked to them. Using a protein-specific label, for example [^{35}S]methionine, has the disadvantage that minute contaminations of non-cross-linked proteins, which may be present even under the most stringent conditions, will give the wrong answers. Furthermore, the amount of label acquired in the cross-linked proteins depends strongly on the methionine content of the protein and on its rate of synthesis and degradation.

In this study we have used the UV cross-linking method to analyze the proteins associated in vivo with adenovirus specific mRNA. Our results show

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that some proteins known to be associated with host cell mRNA, in the infected cell are bound to virus specific mRNA.

2. MATERIALS AND METHODS

HeLa S3 cells were grown in suspension cultures at a density of 2–5 × 10^5 cells ml^{-1} in minimal essential medium (MEM) supplemented with 10% newborn calf serum (Flow Lab. Ltd.). The cells were infected with adenovirus type 2 (about 2000 particles per cell) at a density of 5–20 × 10^6 cells ml^{-1} MEM containing 1 mM arginine. After ad sorption for 1 h at 37°C, the cells were diluted with MEM containing 5% calf serum to a final cell concentration of 3×10^5 ml^{-1} and kept spinning at 37°C. Suspensions of adenovirus infected cells (16 h after infection) and uninfected cells, 2×10^6 cells ml^{-1}, were labeled with [2,5′-8-^{3}H]adenosine (42 Ci/mmol), [5-^{3}H]cytidine (31 Ci/mmol) [8-^{3}H]-guanosine (5 Ci/mmol) and [5,6-^{3}H]uridine (40 Ci/mmol), each 2 μCi/ml for 4 h at 37°C. After labeling the cells were harvested by centrifugation and irradiated with 254 nm light for 3 min (corresponding to a radiation dose of 24 000 J/m^2) as described earlier [6,9].

The cytoplasmic extracts, prepared as described [6] were made 1% in SDS and heated for 2 min at 90°C to dissociate all non-covalent RNA–protein complexes. Polyadenylated RNA and RNA–protein complexes were isolated by oligo(dT)-cellulose chromatography [6]. The low salt eluate from the infected fraction was then subjected to preparative hybridization to adenovirus DNA coupled to Sepharose [8].

Control experiments verifying the specificity of the in vivo cross-linking method and subsequent selection procedure via oligo(dT)-cellulose or adenoDNA–Sepharose have also been described [6,8–10]. The retention of the RNA–protein complexes on the oligo(dT)-cellulose is completely dependent on the integrity of the RNA and is not due to direct binding of protein to the column material [6]. Rehybridization of the material eluted from the adenoDNA–Sepharose always showed that at least 70% was virus-specific (data not shown, see also [8]).

The virus-specific RNA–protein complexes and the covalent host mRNA–protein complexes from the uninfected cells were treated with RNase A (25 μg/ml, Sigma Chem. Co.) and micrococcal nuclease (400 U/ml, P-L Biochem. Inc.) in the presence of 2 mM Ca^{2+} for 1 h at 37°C. The proteins from the resulting mixture were analyzed on 10–18% SDS polyacrylamide gels as described [6]. The RNA-linked proteins were detected by fluorography owing to the ^{3}H-labeled nucleotides still attached to them (1–3 nucleotides per polypeptide [11]).

Analysis of the cross-linked cytoplasmic mRNA on 15–36% sucrose gradients in 10 mM Tris · acetate (pH 7.6), and 0.5% SDS was performed after extensive proteinase K treatment (100 μg/ml in 10 mM Tris · acetate (pH 7.6) and 0.5% SDS for 30 min at 37°C) and phenol–chloroform extraction [6,10].

3. RESULTS AND DISCUSSION

About 50–80% of the cellular mRNA and hnRNA are found to be cross-linked to protein after only 3 min of irradiation. This was found for uninfected [10] as well as adenovirus infected HeLa cells (data not shown). Such a radiation dose does not lead to significant mRNA chain breakage. When total cytoplasmic poly(A)-containing mRNA from irradiated and non-irradiated infected cells was analyzed on sucrose gradients (after proteinase K treatment) no significant differences in sedimentation profile could be seen (fig.1).

This apparently undegraded mRNA, cross-linked to proteins after UV irradiation is for the greater part virus-specific. From various reports on the composition of newly synthesized mRNA from adenovirus infected cells (late phase) it is known that only virus-specific mRNA-sequences reach the cytoplasm and that host sequences remain restricted to the nucleus [8,12]. Analysis of the total newly synthesized population of cross-linked mRNA–protein complexes from virus infected cells thus would seem sufficient to gain insight into possible differences in protein composition between host and virus specific particles.

Although such differences have been reported [3,5] we were unable to confirm these findings using the in vivo UV cross-linking technique. In fact, the pattern of the cross-linked proteins from infected cells (fig.2, lane d) was essentially similar to the pattern found with host cell mRNA–protein

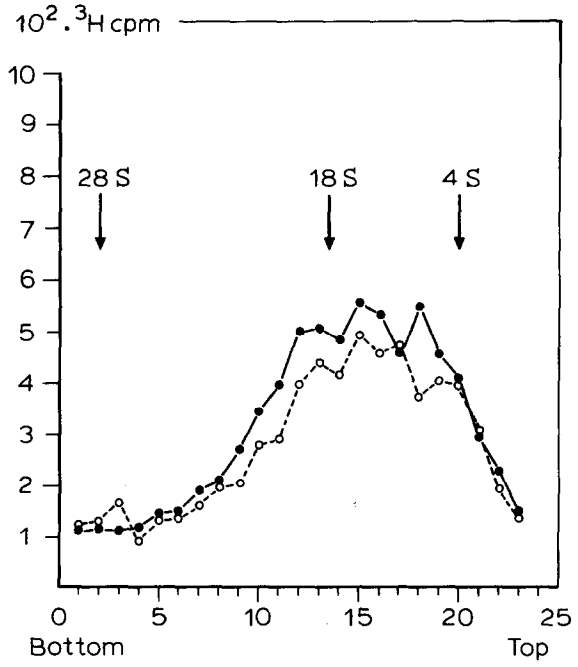

Fig.1. Analysis of poly(A)-containing RNA from irradiated and unirradiated adenovirus infected HeLa cells. Poly(A)-containing cytoplasmic RNA from irradiated and unirradiated adenovirus infected HeLa cells (20 h after infection) was isolated as described in Materials and Methods. Analysis of the RNA was performed on 15–36% sucrose gradient (16 h, 110 000 × *g* in an IEC-SB283 rotor at 20°C). (•—•) RNA from unirradiated infected cells. (○- - -○) RNA from irradiated infected cells. Size markers (*E.coli* tRNA and HeLa cell ribosomal RNAs) were run in a parallel gradient.

complexes (fig.2, lane b). Three proteins with molecular weights of 73 000, 68 000 and 52 000 were most efficiently cross-linked to host mRNA as well as to mRNA from infected cells. Some other cross-linked proteins, not found in the host mRNP fraction, notably the proteins having M_r of 43 000–41 500 and 37 000–35 000 are nuclear contaminants since they comigrate with the major proteins crosslinked to host and viral hnRNA (compare lanes c and d of fig.2). It is known that late after infection with adenovirus the nuclei are much more susceptible to breakage as a result of changes in the nuclear structure and the accumulation of viral particles. Since, under our labeling conditions, the specific activity of hnRNA is much higher than that of cytoplasmic mRNA and, consequently, cross-linked hnRNA-associated proteins will acquire more label than mRNA-associated protein, a relatively small contamination of nuclear fragments will lead to the presence of readily detectable hnRNA-associated proteins. The fact that in the various experiments performed these additional cross-linked proteins were present in varying amounts (in contrast to the very reproducible pattern of the 72 000, 69 000, and 52 000 M_r

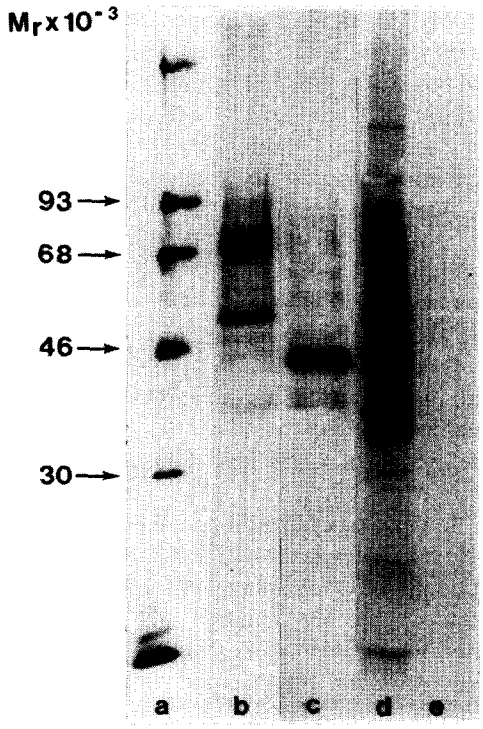

Fig.2. Analysis of proteins cross-linked to mRNA from uninfected and adenovirus infected HeLa cells. Messenger RNA–protein complexes from uninfected and infected cells were prepared and analyzed as described in Materials and Methods. hnRNA–protein complexes from infected cells were isolated as described earlier [8]. Lane a: ^{14}C-labeled marker proteins. M_r values are indicated. Lane b: proteins covalently linked to cytoplasmic mRNA from irradiated uninfected HeLa cells. Lane c: proteins covalently linked to hnRNA from irradiated infected HeLa cells. Lane d: proteins covalently linked to mRNA from irradiated infected HeLa cells. Lane e: proteins covalently linked to mRNA from unirradiated infected HeLa cells.

proteins) also points to possible nuclear contamination.

Since it was not excluded that the presence of labeled host mRNA–protein complexes in our preparation could have obscured the true virus specific pattern, we decided to select and analyze the virus specific mRNA–protein particles. The results of this type of experiment are shown in fig.3. They clearly show that the proteins associated in vivo with virus specific mRNA (fig.3, lane c) are identical with host proteins which in the uninfected cell are bound to host mRNA (fig.3, lane b). The presence of cross-linked viral hnRNA-associated proteins, probably resulting from nuclear contamination as discussed above, is also evident.

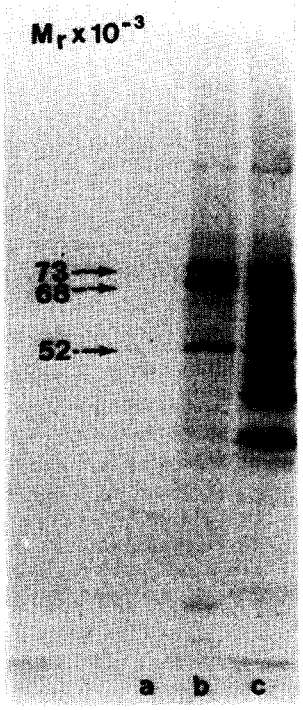

Fig.3. Analysis of proteins cross-linked to host and adenovirus specific cytoplasmic mRNA. HeLa and virus specific mRNA–protein complexes were isolated and analyzed as described in Materials and Methods. Lane a: proteins covalently linked to mRNA from unirradiated infected HeLa cells. Lane b: proteins covalently linked to mRNA from irradiated uninfected HeLa cells. Lane c: proteins covalently linked to virus specific mRNA from irradiated infected HeLa cells. Molecular weights were calculated from the mobilities of ^{14}C-labeled marker proteins (see fig.2).

Using conventional techniques the presence of a tightly bound virally coded 100 K protein in mRNA–protein complexes from adenovirus infected cells has been described [3,5]. Such a protein could not be detected in our cross-linked particles. It is possible that this 100 K protein is not as tightly bound to the mRNA as are the other mRNA-specific proteins or that the association of the 100 K protein with the mRNA sequence is such that no efficient cross-linking can occur. Another explanation, however, would be that the virally coded 100 K protein in vivo is not associated with mRNA but becomes bound during homogenization and isolation procedures.

Our results show that cytoplasmic viral mRNA in vivo is associated with a set of host proteins normally, in the uninfected cell, bound to host mRNA. These proteins are different from the proteins associated with viral hnRNA [8] indicating that during transport of RNA from nucleus to cytoplasm the set of proteins interacting tightly with the RNA changes. Since it has been suggested that cytoplasmic mRNA associated proteins exchange with free proteins [13] it is possible that competition for these mRNA associated proteins between host mRNA and the steadily increasing and finally huge amounts of viral mRNA may be one of the factors involved in the cessation of host protein synthesis late after infection.

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